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13. ABSTRACT *(Maximum 200*

We investigated an N-terminally truncated HER-2/neu product of 95 kDa, for its relationship to shedding of the extracellular domain (ECD) of p185HER-2 and for its association with prognostic factors in human breast cancer. P95 had *in vitro* kinase activity indicating it is active as a signaling protein and its levels correlated with the ECD shed from different cells under varied conditions. Cancer tissues were analyzed by western blotting and scored for p95 and for p185HER-2/neu expression. Of 161 breast cancer tissues, 22.4% expressed p95, 21.7% overexpressed p185, and 14.3% were both p95 positive and overexpressed p185. A higher proportion of node positive patients (23 of 78) than node negative patients (9 of 63) expressed p95 in all tumors combined (P=.032). In the group that overexpressed p185, those that contained p95 were associated with node positive patients (15 of 21), whereas those that were p95 negative were associated with node negative patients (8 of 11) (P=.017). Our findings show that breast cancers, which express the HER-2/neu oncogene, are heterogeneous with respect to HER-2/neu protein products. P95HER-2/neu appears to distinguish tumors that have metastasized to the lymph nodes from those in node negative patients.

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INTRODUCTION

Subject and background: The HER-2/neu gene encodes a receptor-like tyrosine kinase (RTK) which is a member of the epidermal growth factor receptor family (1). Overexpression of HER-2/neu has been observed in tumors arising at many sites including lung (2), colon (3), prostate (4), ovary, and breast (5). In human breast cancer, where HER-2/neu involvement has been extensively studied, overexpression occurs in 15-30% of the cases (see 6) and predicts for significantly lower survival rate and shorter time to relapse in patients with lymph node positive disease (5-8). The significance of HER-2/neu in node negative patients is controversial and so far its clinical utility as a prognostic indicator is limited (8,9). Various approaches are being taken toward HER-2/neu targeted therapeutics many of which are based on antibodies specific to the extracellular domain (ECD) of the transmembrane protein, which either down regulate receptor function or target recombinant toxins with the goal of specifically killing HER-2/neu expressing tumor cells (8,9,10).

In addition to the full length transmembrane product, p185, of the HER-2/neu gene, a truncated product corresponding to the extracellular domain (ECD) is released from breast carcinoma cells in culture by regulated proteolysis (11-13), and is also produced from an alternative transcript (14). HER-2/neu ECD is elevated in the serum of patients with breast (15), ovarian (16), and prostate cancer (17). Several studies of breast cancers estimate that 6% or less of early stage, about 25% of patients with metastatic and locally advanced disease, and greater than 50% of patients with recurrent metastatic disease have elevated serum ECD (see 18). Elevated ECD in serum is associated with overexpression of HER-2/neu in tumor tissue and also reflects tumor load (19-20). Soluble HER-2/neu is a marker of metastatic disease and may predict recurrence (19), shortened survival (20-23), and response to antiestrogen therapy in advanced stage patients (24,25). Serum ECD has also been reported to neutralize the activity of anti HER-2/neu antibodies targeted to the ECD (26,27) possibly allowing escape of HER-2-rich tumors from immunological control.

Proteolytic release of the ECD is expected to create an N-terminally truncated, membrane-associated fragment with kinase activity. Cellular fragments created by ectodomain shedding have been described for the colony stimulating factor receptor (CSF-1R) (28), the TrkA neurotrophin receptor (29), Axl receptor (30), and HER-4 (31), while a truncated cellular product of HER-2/neu shedding has not yet been identified. The truncated CSF-1R was found to have *in vitro* kinase activity (28), and the cytoplasmic HER-4, induced by phorbol ester tumor promoters, had little or no kinase activity (31) while a truncated HER-4 found in cells treated with a proteosome inhibitors was an active kinase (32).

Several lines of evidence indicate that the ECD of full length transmembrane receptors exerts a negative regulatory constraint on their signaling activity. Engineered deletion of a region of the HER-2 ECD was found to enhance its oncogenic potency (33-36). This has been

illustrated by engineered removal of the ECD from the epidermal growth factor (EGF) receptor and by the oncogenic potency of viral encoded v-erbB, v-kit, and v-ros, that are missing regions of the ECD found in their normal cellular counterparts (37). Naturally occurring mutant EGF receptors with N-terminal truncations have been identified in several human carcinomas (38) and have constitutive signaling activity and enhanced oncogenic transforming activity in cell culture and animal models (39,40).

Purpose and scope: The purpose of the project was to investigate the signaling activity and prognostic significance of variant forms of p185HER-2 in breast cancer. In particular, we sought to investigate the signaling activity and possible prognostic signficance of the N-terminally truncated HER-2 product, hypothesized to be the cytoplasmic tail of shedding.

<u>Contains proprietary information</u>

BODY

Following is a revised SOW submitted to the Army last year: We have listed the task as originally proposed and then described the modifications and results that were obtained. This revised SOW was approved. All figures are in the manuscript in the appendix.

Task 1 Months 1-12: *Further characterizations of selected samples and their variant forms will be conducted.* Originally this was to be conducted on cancer tissues in a 3 month period. However additional time was needed to further characterize the HER-2/neu products in cultured cells first before going to the cancer tissues. Reviewers of our manuscript requested additional data on the structure and function of the p95 kinase domain (originally called p80) in cultured cells, its correlation with shedding of the ECD, evidence of its signalling activity, and additional evidence that it was created by proteolytic processing in intact cells.

SIGNALLING ACTIVITY OF P95

N-terminally truncated p95 has kinase activity. HER-2 transfected cells, 17-3-1, were extracted, resolved in gels, and immunoblotted with antibodies against the C-terminus of the HER-2/neu product [anti-neu(C)]. Two major protein products were detected in cell extracts; the full length p185 HER-2/neu and a truncated protein of about 95 kDa (Fig.1, lane 1). Extracts were immunoprecipitated and the 95kDa protein as well as p185HER-2/neu were phosphorylated in the immune complex with $(\gamma\text{-}^{32}P)ATP$ (Fig.1, lane 2). A monoclonal antibody specific for the N-terminal region of p185HER-2/neu [anti-neu(N)] did not immunoprecipitate p95 indicating that the N-terminal region was missing (lane 3). To examine whether p95 had self-phosphorylating activity or was a substrate of the full length receptor tyrosine kinase, p185 was first removed from the cell lysate with anti-neu(N), and then p95 was immunoprecipitated with anti-neu(C). P95 was phosphorylated when p185 levels were greatly depleted (Fig.1 lane 4) indicating that it has kinase activity.

P95 kinase activity is in human breast carcinoma cells but not in nontumorigenic breast epithelial cells. The breast carcinoma cell line, BT474, known to release soluble ECD (11) also contained two autophosphorylated HER-2/neu products, p185 and p95, which were at elevated levels compared to the nontumorigenic breast epithelial cell line HBL-100 (Fig. 2). It

was possible that p95 could not be detected in the small amount of HBL-100 cells, since they express low levels of HER-2 (43). To compensate for different levels of HER-2/neu expression (43), the amounts of extract from HBL-100, human mammary epithelial cells, (HMEC), and three breast carcinoma cell lines were adjusted and proteins were phosphorylated with $(\gamma\ ^{-32}P)$ ATP. P95 was detected in the low (MDA-MB-453) and high (BT474 and SKBR3) HER-2/neu expressing breast carcinoma cells, but not in the HBL-10 nor HMEC cells, despite a robust signal from the HER-2/neu receptor which migrated as a slightly smaller protein in the breast epithelial cells (Fig. 2).

P95 is tyrosine phosphorylated and in the membrane fraction from BT474 cells. Tyrosine phosphorylation of tyrosine kinase receptors indicates their activation in signaling (9,10). The tyrosine phosphorylation of p95, and its subcellular location were examined by fractionation of BT474 cell extracts into a soluble fraction and a particulate fraction which were immunoprecipitated with anti-neu(C) and then subjected to western blot analysis using monoclonal antibodies against phosphotyrosine. Figure 3 illustrates that tyrosine phosphorylated p95 fractionated with p185 in the particulate fraction which contains the plasma membranes. P95 was further shown to be tyrosine phosphorylated by first immunoprecipitating with anti-phosphotyrosine antibodies and then probing the western blot with anti-neu(C).

P95 CORRELATES WITH SHEDDING OF THE ECD

P95 correlates with shedding of the ECD from different cells treated with TPA and chloroquine. To examine the relationship of p95 to soluble ECD, their levels were compared in different cells under varied conditions. The basal levels of ECD and cellular p95HER-2/neu were first examined in two cell lines that overexpress HER-2/neu, BT474 and the ovarian carcinoma cell line SKOV-3, which was previously reported to produce low levels of ECD (13). The amount of p95 relative to p185 and to cell protein was greatly elevated in BT474 cells and correspondingly, the ECD in the extracellular medium from BT474 cells, detected with anti-neu(N), was enhanced by greater than 10 fold compared to the SKOV3 cells (Fig.4).

Shedding of several membrane proteins is rapidly and transiently induced by phorbol ester tumor promoters (44,45). While short term treatment with tumor promoters does not induce HER-2 shedding (31), chronic administration of the phorbol ester TPA synergizes with chloroquine to stimulate release of soluble HER-2 (Christianson, Lin, and Clinton, unpublished observations). To determine whether p95 and ECD were coordinately regulated, TPA (500 nM) and chloroquine (50 μM) or the control vehicle were added to the culture media of BT474 and SKOV3 cells. At 24 hrs, the ECD levels in the extracellular media and p95 levels in the cell extract were analyzed. Soluble ECD was elevated several fold in the conditioned medium from stimulated BT474 cells and SKOV3 cells, while p95 was upregulated about three-fold in BT474 cells (Fig.4). Overexposure of the immunoblot revealed that p95 in SKOV3 cell extracts was also stimulated about three-fold by TPA and chloroquine (data not shown).

A metalloprotease inhibitor depresses levels of p95 and ECD from BT474 cells. The shedding of diverse transmembrane proteins has been found to be inhibited by hydroxamic

acid-based compounds, which are potent metalloproteinase inhibitors (46-48). We therefore tested the effects of different concentrations of the hydroxamic acid, TAPI (47) on shedding of HER-2/neu ECD and on cell levels of p95. TAPI at 0 to 40 μM was added to cultured BT474 cells for 24 hrs, the ECD in concentrated conditioned media was analyzed by immunoblotting with anti-neu(N), and p95 and p185 were examined in cell extracts using anti-neu(C). The results in Figure 5 show that production of ECD was partially inhibited at 1μM TAPI and maximally inhibited by 10 μM TAPI. A residual amount of about 10% of the ECD resisted inhibition by 40 μM TAPI. The level of truncated p95 in the cytoplasm was also inhibited by TAPI, with little or no effect at 1 μM and maximal inhibition at 10 μM (Fig.5). In three separate experiments, 1μM TAPI inhibited ECD and p95 levels by 50% or less, and in all cases, maximum inhibition was achieved by 10 μM of TAPI. No change in p185HER-2/neu levels could be detected in cells treated with TAPI or when shedding was stimulated by TPA and chloroquine (Fig.4) probably because proteolytic processsing of p185 is constitutive and limited with about 20% converted into soluble HER-2/neu in 2 hrs (13).

In vitro kinase activity of p95 in breast cancer tissue. As originally proposed, we characterized tissue samples by immunoprecipitations to detect autophosphorylation activity of HER-2/neu variant products. We subsequently gave up this approach because we could not extract products that retained their enzyme activity from available tumor tissues.

Task 2: Months 4-7: *Additional characterizations providing new information on these samples.* This task was modified and basically incorporated into Task 1. Immunohistochemical examination of tissue samples did not reveal distinct subcellular locations of HER-2/neu reactive proteins when the samples contained large amounts of the kinase domain. Further, the task of coimmunoprecipitations with active signaling molecules such as phospholipase C could not be completed since prohibitively large amounts of tissue were required to observe the targeted proteins by the technique of coimmunoprecipitation.

Task 3 Months 8-16. This task involved developing techniques to analyze and quantitate HER-2 proteins in breast cancer tissues.

Analysis of p95HER-2 in breast cancer tissue. To establish a standard for quantitation of HER-2/neu variant forms in western blots of breast cancer tissues, we first analyzed a variety of tumor tissue to define a group that overexpressed HER-2/neu using an ELISA immunoassay approach. Aliquots of membrane-rich fractions prepared from breast cancer tissue were assayed using the Triton Diagnostics c-erbB-2 Tissue Extract EIA kit (Ciba Corning). This assay employed two monoclonal antibodies against the HER-2/neu ECD. The HER-2/neu units/mg protein in the specimens were calculated from a calibration curve generated by plotting the HER-2/neu concentration of the calibration standards versus the absorbance obtained from the immunoassay. To define the samples that overexpressed p185HER-2/neu, specimens with HER-2 immunoassay values that were considered HER-2/neu-rich (400 units or greater) compared to samples with low HER-2/neu levels (less than 400 units) were characterized for their p185 signal. Once several samples that overexpressed HER-2/neu were defined based on ELISA, these samples were compared with control standard cell extracts to

define an internal standard to be used for the entire study to analyze multiple tumor tissue. We chose the control cells, 17-3-1, which are transfected and overexpress HER-2/neu. Comparisons of the control cell extracts with tumor tissue found to overexpress HER-2/neu by ELISA revealed that a signal for p185 that was greater than or equal to the signal from the control cell extracts defined the group that overexpressed HER-2/neu. Those samples with a p185 signal that could be detected by 1 min exposure of the membrane to film and that was equal to or greater than the p185 level found in 3 μg of 17-3-1 cells, as revealed by laser densitometric analysis of the film, were scored as highly positive. Twenty μg of protein from the membrane fraction prepared from each tumor sample were resolved under denaturing and reducing conditions by SDS-PAGE in 10% gels. Each gel also contained 3 μg of protein from extracts of 17-3-1 cells to mark the migration of p185 and p95 and to provide a standard for the entire study. Proteins were electrotransferred onto membranes as described above, which were incubated with anti-neu(C) diluted 1:10,000 in TBS-Tween at 4°C overnight with shaking and then incubated with a 1:10,000 dilution of goat anti-rabbit HRP conjugated antibody (Bio-Rad) for 40 min at room temperature. To develop the blot, the membranes were incubated with chemilumenescent reagent and exposed to film for 1, 5, 20, and 120 min.. In the samples that had detectable p95, its level ranged from 10% to 100% of p185. Specimens were scored as positive if p95 was detected at a 10% or greater proportion of p185 by 2 hrs of exposure of the membrane to film. Because of the high titer of the primary antibody, anti-neu(C), there were rarely any background bands even when the immunoblots were exposed to film for 2 hrs. It took additional time to develop and characterize the ELISA test.

Task 4 months 16-22: Breast cancer samples (about 600) will be pulverized, aliquoted, extracted and protein amounts determined. This task was similar except that the time frame was longer than expected and fewer breast cancers were analyzed. This step took longer because we had to conduct additional fractionation steps on the breast cancer tissues. These additional steps were required because of the contamination with extracellular matrix components which diluted the breast cancer tissues to be analyzed. These fractionations required examining HER-2/neu proteins at each step of the procedure to define the fractionation properties. In addition, fewer samples were analyzed than originally expected.

p95HER-2 IS ASSOCIATED WITH LYMPH NODE METASTASIS IN BREAST CANCER.

Detection of p185 and p95 HER-2/neu in breast cancer tissue. Tumor tissues were homogenized, fractionated, and examined for HER-2/neu proteins by western analysis. The membrane-enriched but not the soluble fraction (data not shown) from some tumor tissues contained the full length product, p185, and the truncated p95HER-2/neu protein that comigrated with HER-2/neu proteins from the control 17-3-1 cells (Fig. 6). In addition, p95, along with p185, was detected in 2 of 8 ovarian cancer tissues (data not shown). Initial analyses of several breast cancer tissues revealed distinct expression patterns of p95 and p185. One group had no detectable p185 or p95 (see # 39 and 69, Fig. 6). A second category of specimens expressed both p185 and p95 (#60,53,04,22). An additional group contained p185

with relatively little or no p95 (#40,58,38,57,17,75). As observed in previous studies, some samples were p185-rich (#04,22,57,17,75). The samples that were characterized as highly positive for p185 were initially identified by immunoassay values of greater than 400 units (see Methods and legend to Fig. 6). The results of the western analysis suggested that the tumors were heterogeneous with respect to HER-2/neu protein products and that they could be subdivided based on the presence or absence of p95.

Western analysis of 161 breast cancer samples revealed that 22.4% were p95 positive. The p185 positive samples were further subdivided into highly positive or HER-2-rich specimens based on comparisons with HER-2/neu overexpressing samples identified by immunoassay and comparisons with the control 17-3-1 extract as described in Methods. The highly positive p185 samples represented 21.7% of the total. All samples that expressed p95 were also positive for p185, although 65% of p185 positive samples did not contain p95. Of the p95 positive samples, 63.9% were also highly positive for p185 and 36% had low p185 levels.

Relationship between p95 positive, p185 highly positive, and other prognostic factors. Of 78 node-positive breast cancer patients, a higher proportion expressed p95, than for the node negative patients (P=.032), while p185 rich samples had no significant association with node status (Table 1). Neither p95 positive nor p185 rich samples correlated significantly with other factors known to predict poor prognosis (49) including estrogen receptor and progesterone receptor negativity or tumor size of 3 cm or greater (Table 1).

Influence of p95 in the p185 highly positive group. We questioned why a similar percentage of node positive and node negative patients were p185-rich (24.4% versus 22.2%, Table 1), while p95 was associated with node positive patients, since 65.7% of the p185-rich samples contained p95. We therefore examined whether the presence or absence of p95 in the specimens that overexpressed p185HER-2/neu affected the relationship with lymph node status (Table 2). The p185 highly positive samples that contained p95 (n=21) had a significantly higher association with metastasis to the lymph nodes, while the p185 highly positive samples that were negative for p95 (n=11) were associated with lymph node negative patients (P=.017).

Table 1 *Relationship between p95 positive, p185 highly positive, and other prognostic factors[a]*

Factor	% p95 Positive	P value	% p185 High Positive	P value
Nodes		.032		NS[b]
Pos(78)	29.5		24.4	
Neg(63)	14.3		22.2	
Tumor Size		NS		NS
≥3cm(54)	27.8		22.2	
<3cm(79)	17.7		21.5	
ER		NS		NS
Neg(37)	32.0		29.7	
Pos(117)	19.7		17.9	
PR		NS		NS
Neg(59)	23.7		20.3	
Pos(95)	22.1		23.2	

[a] 161 samples were examined by western analysis. See Methods for a description of patient material used and methods of analysis. Not all samples had information for the factors examined.

[b] NS = not significant.

Table 2 *Relationship between p185 highly positive samples that are p95 negative versus p95 positive with node status.*

	p185 highly positive[a]	
	p95 positive n=21	p95 negative n=11
node positive	71.4%[b]	27.3%
node negative	28.6%	72.7%

[a] The p185 highly positive group (n=32) was divided into those that contained p95 (n=21) and those that were p95 negative (n=11).

[b] The samples that contained p95 had a significantly higher association with node positive patients (15 of 21), and those that were p95 negative correlated with node negative patients (8 of 11) (P=.017).

For task 4, we also proposed to examine the signaling activity and prognostic significance of the LBDp-100 variant thought to be an intracellular form of the HER-2 ECD. Our studies revealed that his product was difficult to analyze, since it required each sample to be run on an additional gel under nonreducing conditions for detection with the monoclonal antibody. Moreover, there was not a biological rationale to examine its role in breast cancer since this product should be secreted based on missing its transmembrane domain. Additional studies found that this product accumulated in the endoplasmic reticulum and was not fully processed. Therefore, our studies focussed on the truncated kinase domain because of the strong biological rationale, and based on its association with shedding of the HER-2/neu extracellular domain from cultured cells.

Task 5 months 20-22. Films will be scanned, standard curves prepared, and variant forms will be quantitated. Patients subsets will be defined based on patterns of variant expression. This task remained the same except was moved to a later period in the funding cycle corresponding to the added time required to characterize the variant forms and to fractionate and analyze tumor tissues. This task was accomplished as described above.

Task 6 months 20-24: Patients subgroups will be statistically correlated with disease stage at surgery, histological subtype of tumor and pathologic factors. Originally this task included patient outcome. However, because of the added complexities described above, this task was refocussed to correlated p185HER-2/neu and truncated kinase domain expression with pathologic factors including tumor size, hormone receptor status, lymph node involvement, and stage of the disease.

In summary. We found a significant correlation of the p95 kinase domain with lymph node metastasis. In regard to our orignial aims and statement of work, we had to modify the time frame and the procedures to be employed. In addition, to publish our results, it was necessary to conduct additional analyses that were not anticipated when the original SOM was developed. We were successful in the final outcome of identification of an additional marker based on a HER-2/neu variant form.

CONCLUSIONS

In conclusion, it has been difficult to understand why HER-2/neu overexpression occurs in tumor tissue from both node negative and positive patients, yet it is a strong prognostic marker only in node positive patients. Our findings indicate that p95 is preferentially found in HER-2/neu positive patients with lymph node involvment. It is possible that higher expression of p95 is a critical factor that helps explain the increased prognostic significance of HER-2/neu in node positive patients. It will be of interest and importance to examine the prognostic significance of p95 particularly in node negative patients.

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APPENDICIES

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Bibliography:

Publication
Tracy A. Christianson, Joni K. Doherty, Yuzhong J. Lin, Elizabeth E. Ramsey, Ron Holmes, Edward J. Keenan, and Gail M. Clinton N-terminally truncated HER-2/neu protein: Relationship with shedding of the extracellular domain and with prognostic factors in breast cancer.*Cancer Research* (1998) in press.

Abstract
DOD "Era of Hope" Breast Cancer Meeting. October, 1997

Gail M. Clinton, Tracy A. Christianson, Joni Koherty, Ron Holmes, Edward Keenan, and Juzhong J. Lin. Truncated HER-2/neu cytoplasmic domain with kinase activity in breast cancer.

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NH₂-terminally Truncated HER-2/neu Protein: Relationship with Shedding of the Extracellular Domain and with Prognostic Factors in Breast Cancer[1]

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ABSTRACT

We identified an NH₂-terminally truncated HER-2/neu product of M_r 95,000 with in vitro kinase activity by Western blotting and immunoprecipitations using domain-specific antibodies. p95 levels correlated with the extracellular domain (ECD) shed from different cells under varied conditions. Both ECD and p95 were at ~20-fold lower levels in SKOV3 ovarian carcinoma cells, as compared to BT474 breast carcinoma cells. Both were stimulated by treatment of cells with the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the lysosomotrophic agent chloroquine. The hydroxamate inhibitor of metalloproteases, TAPI, suppressed both p95 and ECD in a dose-dependent fashion, with maximal inhibition at ≤10 μM in BT474 cells. Cancer tissues were analyzed by Western blotting and scored for p95HER-2/neu and for p185HER-2/neu expression. Breast and ovarian cancer tissues were both found to express p95HER-2/neu in addition to p185HER-2/neu. Of 161 breast cancer tissues, 22.4% expressed p95, 21.7% overexpressed p185, and 14.3% were p95 positive and overexpressed p185. A higher proportion of node-positive patients (23 of 78) than node-negative patients (9 of 63) expressed p95 in all tumors combined (P = 0.032). In the group that overexpressed p185, those that contained p95 were associated with node-positive patients (15 of 21), whereas those that were p95 negative were associated with node-negative patients (8 of 11; P = 0.017). Neither p95- nor p185-rich patients significantly correlated with tumor size or with hormone receptor status in this study. Our findings show that breast cancers, which express the HER-2/neu oncogene, are heterogeneous with respect to HER-2/neu protein products. p95HER-2/neu appears to distinguish tumors that have metastasized to the lymph nodes from those in node-negative patients.

INTRODUCTION

The HER-2/neu (erbB-2) gene encodes a RTK,[5] which is a member of the EGF receptor family (1). Overexpression of HER-2/neu has been observed in tumors arising at many sites, including non-small cell lung (2), colon (3), prostate (4), ovary, and breast (5). In human breast cancer, in which HER-2/neu involvement has been extensively studied, overexpression occurs in 15–30% of the cases (see Ref. 6) and predicts a significantly lower survival rate and a shorter time to relapse in patients with lymph node-positive disease (5–8). The significance of HER-2/neu in node-negative patients is controversial, and thus far, its clinical utility as a prognostic indicator is limited (8, 9). Various approaches are being taken toward HER-2/neu-targeted ther-

apeutics many of which are based on antibodies that are specific to the ECD of the transmembrane protein, which either down-regulate receptor function or target recombinant toxins, with the goal of specifically killing HER-2/neu-expressing tumor cells (8–10).

In addition to the full-length transmembrane product of the HER-2/neu gene, p185, a truncated product corresponding to the ECD is released from breast carcinoma cells in culture by regulated proteolysis (11–13) and is also produced from an alternative transcript (14). HER-2/neu ECD is elevated in the serum of patients with breast (15), ovarian (16), and prostate (17) cancer. Several studies of breast cancers estimate that ≤6% of patients with early-stage disease, ~25% of patients with metastatic and locally advanced disease, and >50% of patients with recurrent metastatic disease have elevated serum ECD (see Ref. 18). Elevated ECD in serum is associated with overexpression of HER-2/neu in tumor tissue and also reflects tumor load (19, 20). Soluble HER-2/neu is a marker of metastatic disease and may predict recurrence (19), shortened survival (20–23), and response to antiestrogen therapy in advanced-stage patients (24, 25). Serum ECD has also been reported to neutralize the activity of anti-HER-2/neu antibodies targeted to the ECD (26, 27), possibly allowing escape of HER-2-rich tumors from immunological control.

Proteolytic release of the ECD is expected to create an NH₂-terminally truncated, membrane-associated fragment with kinase activity. Cellular fragments created by ectodomain shedding have been described for the colony-stimulating factor receptor (28), the TrkA neurotrophin receptor (29), the Axl receptor (30), and HER-4 (31), but a truncated cellular product of HER-2/neu shedding has not yet been identified. The truncated colony-stimulating factor receptor was found to have in vitro kinase activity (28), and the cytoplasmic HER-4, induced by phorbol ester tumor promoters, had little or no kinase activity (31), whereas a truncated HER-4 found in cells treated with a proteosome inhibitor was an active kinase (32).

Several lines of evidence indicate that the ECD of full-length transmembrane receptors exerts a negative regulatory constraint on their signaling activity. The engineered deletion of a region of the HER-2 ECD was found to enhance its oncogenic potency (33–36). This has also been illustrated by engineered removal of the ECD from the EGF receptor and by the oncogenic potency of viral encoded v-erbB, v-kit, and v-ros, that are missing regions of the ECD found in their normal cellular counterparts (37). Naturally occurring mutant EGF receptors with NH₂-terminal truncations have been identified in several human carcinomas (38) and have constitutive signaling activity and enhanced oncogenic transforming activity in cell culture and animal models (39, 40).

Here, we sought to identify and characterize the NH₂-terminally truncated HER-2/neu protein and examine its correlation with ECD shedding and association with breast cancer pathological factors.

MATERIALS AND METHODS

Cells and Antibodies. Cell lines were obtained from the American Type Culture Collection (Manassas, VA), except the 3T3 cells transfected with HER-2/neu cDNA (17-3-1; provided by Applied BioTechnology, Inc., Cambridge, MA), and the HMECs (provided by Dr. Gary Shipley, Portland, OR)

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were cultured from tissue obtained from reduction mammoplasty. Antipeptide antibody against the COOH terminus of p185HER-2/neu, anti-neu(C), has been described previously (41). Monoclonal antibody against the ECD of HER-2/neu was prepared as described (42) and was provided by Applied BioTechnology, Inc.

Cell Culture. 17-3-1 cells were cultured in DMEM supplemented with 5% fetal bovine serum containing 0.4 mg/ml geneticin (G418; Life Technologies, Inc.). The human breast carcinoma cell line BT474 was cultured in RPMI medium supplemented with 10% FBS and 10 μg/ml insulin. All other cell lines were grown in DMEM supplemented with 10% FBS and the antibiotic gentamicin at 0.05%.

Immunoprecipitations and Immune Complex Kinase Assays. Freshly prepared cell lysates in TEDG buffer [50 mM Tris, 1.5 mM EDTA, 0.5 mM DTT, 10% glycerol (pH 7.5) with 1% aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 2 mM vanadate) containing 1% NP40 were immunoprecipitated by incubation with antibody for 2 h with continuous shaking at 4°C as described (41). The immune complexes, bound to protein G-Sepharose (Pharmacia), were washed twice with TEDG buffer and incubated 10 min on ice in a kinase reaction mixture containing 20 mM HEPES (pH 8.0), 2 mM DTT, 25 μM vanadate, 0.5% NP40, 10 mM MnCl$_2$, 1 μM ATP, and 15 μCi of [γ-^{32}P]ATP (New England Nuclear). The immune complexes were washed three times with buffer, and the proteins were released by boiling for 2 min in SDS-polyacrylamide sample buffer.

Western Blotting. Following SDS-PAGE, cell lysates or proteins from concentrated, conditioned medium were electroblotted onto nitrocellulose (Trans-Blot; Bio-Rad) using a semidry transfer unit (Bio-Rad) at 15 V for 20 min per mini gel of 0.75-mm thickness (Mini-PROTEAN II electrophoresis cell; Bio-Rad) equilibrated with 25 mM Tris (pH 8.3), 192 mM glycine, 50 mM NaCl, and 20% methanol. Binding sites were blocked by incubating the membrane with 5% nonfat dry milk. After incubation with the primary antibody, the blot was washed twice for 15 min each and four times for 5 min each with TBS containing 0.05% Tween and then incubated for 40 min with goat antirabbit or goat antimouse antibody conjugated to horseradish peroxidase (Bio-Rad) diluted in TBS-Tween. After incubation with secondary antibody, the blot was washed as described above with TBS-Tween and developed with chemiluminescent reagent (Pierce).

Cancer Tissue Extraction and Fractionation. About 0.1 g of tumor tissue, which had been fresh-frozen and stored at −70°C, was minced on dry ice and suspended in TEDG buffer. Tissues were homogenized using a Brinkman polytron for 5–10-s bursts repeated two to three times with a chilled probe. Homogenates were centrifuged at 1500 × g for 10 min at 4°C. The lipid layer was removed with a wooden stick, and the supernatant was centrifuged for 20 min at 40,000 × g at 4°C. The lipid layer was collected with a wooden stick, the supernatant was decanted, and the pellet containing the membranes was solubilized in TEDG buffer containing 0.1% SDS for 20 min with intermittent vortexing and clarified by centrifugation at 15,000 × g for 15 min. The protein concentration in the supernatant was determined by the Bio-Rad protein assay reagent, and aliquots were frozen at −80°C.

Analysis of p95 and p185 in Breast Cancer Tissues. Twenty μg of protein from the membrane fraction prepared from each tumor sample were resolved under denaturing and reducing conditions by SDS-PAGE in 10% gels. Each gel also contained 3 μg of protein from extracts of 17-3-1 cells to mark the migration of p185 and p95 and to provide a standard for the entire study. Proteins were electrotransferred onto membranes as described above, which were incubated with anti-neu(C) diluted 1:10,000 in TBS-Tween at 4°C overnight with shaking and then incubated with a 1:10,000 dilution of goat antirabbit horseradish peroxidase-conjugated antibody (Bio-Rad) for 40 min at room temperature. To develop the blot, the membranes were incubated with chemiluminescent reagent (Pierce) for 5 min and then exposed to Kodak X-OMAT AR film for 1, 5, 20, and 120 min. To define the samples that overexpressed p185HER-2/neu, specimens with HER-2 immunoassay values that were considered HER-2/neu-rich (400 units or greater) compared to samples with low HER-2/neu levels (<400 units) were characterized for their p185 signal relative to the control 17-3-1 cells by Western analysis. Those samples with a p185 signal that could be detected by a 1-min exposure of the membrane to film and that was more than or equal to the p185 level found in 3 μg of 17-3-1 cells, as revealed by laser densitometric analysis of the film, were scored as highly positive. Using this method, we identified 21.7% of the samples that overexpressed p185. This proportion is comparable to the 15–

30% of breast cancers found to overexpress HER-2/neu in numerous clinical studies (5–10). In the samples that had detectable p95, its level ranged from 10 to 100% of that of p185. In this pilot study, specimens were scored as positive if p95 was detected at a ≥10% proportion of p185 by 2 h of exposure of the membrane to film. Because of the high titer of the primary antibody, anti-neu(C), there were rarely any background bands, even when the immunoblots were exposed to film for 2 h.

HER-2/neu Tissue Extract ELISA. Aliquots of membrane-rich fractions prepared from breast cancer tissue as described above were assayed using the Triton Diagnostics c-erbB-2 Tissue Extract EIA kit (Ciba Corning) according to manufacturer's instructions. This assay uses two monoclonal antibodies against the HER-2/neu ECD. The HER-2/neu units per milligram of protein in the specimens were calculated from a calibration curve generated by plotting the HER-2/neu concentration of the calibration standards *versus* the absorbance obtained from the immunoassay.

Clinical Data. This investigation of human tissues was reviewed and approved by the Institutional Review Board Committee on Human Research. A computer database contains clinical information on each patient, coded to protect the individual's identity, and includes age, nodal status, size of the primary tumor, age of the patient, stage of disease at diagnosis, ER levels, and PR receptor levels. Specimens were considered ER positive and PR positive if they contained at least 10 fmol of specific binding sites per milligram of cytosolic proteins. The stages of the specimens were as follows: 1 stage 0, 32 stage I, 56 stage II, 45 stage III, and 13 stage IV specimens. Fourteen specimens were of unknown stage. The average age of the patients was 60 years. The eight ovarian cancer tissues included three that were grade III and five that were grade IV.

RESULTS

Identification of NH$_2$-terminally Truncated HER-2/neu Protein with Kinase Activity. 3T3 cells transfected with HER-2/neu cDNA (17-3-1 cells) release soluble ECD by proteolytic processing of p185HER-2/neu (12). To detect truncated cytoplasmic products, we resolved 17-3-1 extracts in gels and immunoblotted with antibodies against the COOH terminus of the HER-2/neu product [anti-neu(C)]. Two major protein products were detected in cell extracts: the full-length p185HER-2/neu and a truncated protein of M_r ~95,000 (Fig. 1, Lane 1). Extracts were immunoprecipitated, and the M_r 95,000 protein as well as p185HER-2/neu were phosphorylated in the immune complex with [γ-^{32}P]ATP (Fig. 1, Lane 2). A monoclonal antibody that was specific for the NH$_2$-terminal region of p185HER-2/neu [anti-
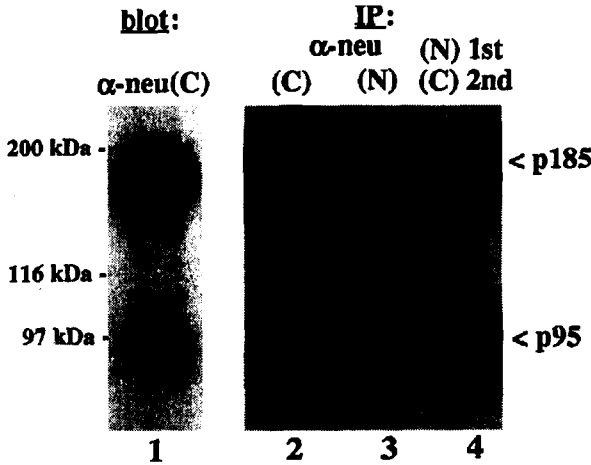

Fig. 1. NH$_2$-terminally truncated HER-2/neu product with kinase activity. Twenty-five μg of protein from 17-3-1 cells were Western blotted with anti-neu(C) diluted 1:10,000 (Lane 1). In Lanes 2–4, 400 μg of protein were immunoprecipitated with anti-neu(C) (Lanes 2 and 4) or with monoclonal antibody against the ECD, anti-neu(N) (Lane 3), or were depleted of p185HER-2/neu by extracting twice with anti-neu(N) and then immunoprecipitated with anti-neu(C) (Lane 4). The immune complexes were phosphorylated with [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography.

2

TRUNCATED HER-2/*neu* KINASE AND BREAST CANCER

neu(N)] did not immunoprecipitate p95, indicating that the NH_2-terminal region was missing (*Lane 3*). To examine whether p95 had self-phosphorylating activity or was a substrate of the full-length RTK, p185 was first removed from the cell lysate with anti-neu(N), and then p95 was immunoprecipitated with anti-neu(C). p95 was phosphorylated when p185 levels were greatly depleted (Fig. 1, *Lane 4*), indicating that it has kinase activity.

p95 Kinase Activity Is in Human Breast Carcinoma Cells but not in Nontumorigenic Breast Epithelial Cells. The breast carcinoma cell line, BT474, known to release soluble ECD (11) also contained two autophosphorylated HER-2/neu products, p185 and p95, which were at elevated levels compared to the nontumorigenic breast epithelial cell line HBL-100 (Fig. 2). It was possible that p95 could not be detected in the small amount of HBL-100 cells because they express low levels of HER-2 (43). To compensate for different levels of HER-2/neu expression (43), the amounts of extract from HBL-100, HMECs, and three breast carcinoma cell lines were adjusted, and proteins were phosphorylated with $[\gamma\text{-}^{32}P]$ATP. p95 was detected in the low (MDA-MB-453) and high (BT474 and SKBR3) HER-2/neu-expressing breast carcinoma cells but not in the HBL-100 nor HMEC cells, despite a robust signal from the HER-2/neu receptor, which migrated as a slightly smaller protein in the breast epithelial cells (Fig. 2).
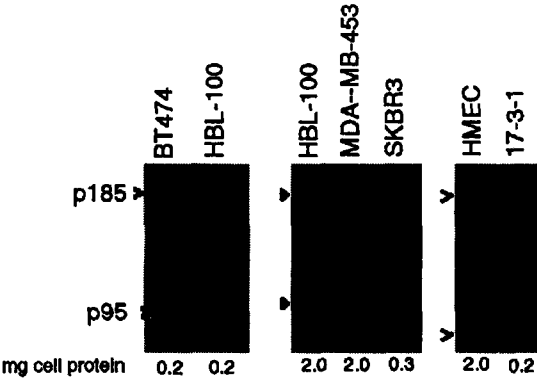
Fig. 2. Human breast carcinoma cell lines contain p95HER-2/neu. Indicated amounts of cell lysates from BT474, HBL-100, MDA-MB-453, SKBR3, HMEC, and 17-3-1 cells were immunoprecipitated with anti-neu(C) and phosphorylated as in Fig. 1.
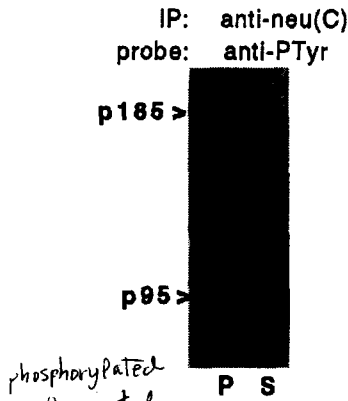
Fig. 3. Tyrosine phosphorylation of p95 localized in the particulate fraction of BT474 breast carcinoma cells. Particulate (*Lane P*) and soluble (*Lane S*) fractions were prepared by incubation of 10^7 cells in ice for 10 min in 3 ml of 10 mM Tris (pH 7.4), 10 mM NaCl, and 2 mM MgCl₂ with 2 mM vanadate and protease inhibitors, Dounce homogenization, and centrifugation at 100,000 × g for 1 h. The pellet was resuspended in 3 ml of homogenization buffer, and 200 μg of protein from the particulate fraction and an equal volume of the soluble fraction were immunoprecipitated with anti-neu(C) and analyzed as a Western blot with monoclonal antiphosphotyrosine antibody (Sigma Chemical Co.).
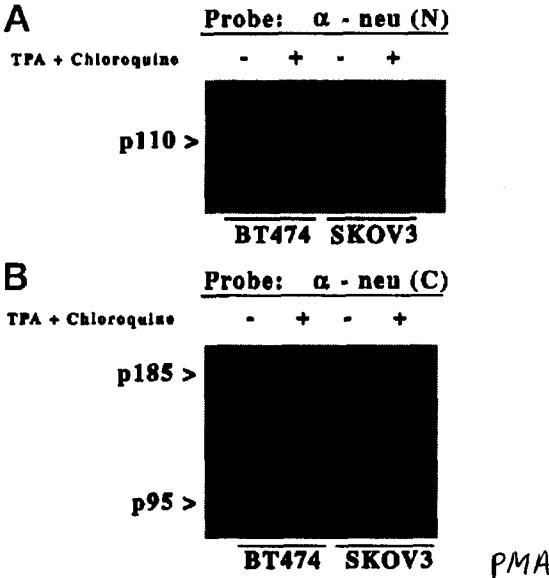
Fig. 4. Expression of p95 and ECD in SKOV3 and BT474 cells. Cells were treated for 24 h in serum-free medium with control vehicle or with 500 nM phorbol ester,TPA and 50 μM chloroquine. A, 5 ml of conditioned media were concentrated 100-fold, denatured under nonreducing conditions, and aliquots normalized to cell extract protein were analyzed by Western blotting with anti-neu(N) monoclonal antibody at 1 μg/ml. B, 20 μg of cell proteins were analyzed by Western blotting using anti-neu(C). The results are representative of three replicate experiments.

p95 Is Tyrosine-phosphorylated and Is in the Membrane Fraction from BT474 Cells. Tyrosine phosphorylation of tyrosine kinase receptors indicates their activation in signaling (9, 10). The tyrosine phosphorylation of p95 and its subcellular location were examined by fractionation of BT474 cell extracts into a soluble fraction and a particulate fraction, which were immunoprecipitated with anti-neu(C) and then subjected to Western blot analysis using monoclonal antibodies against phosphotyrosine. Fig. 3 illustrates that tyrosine-phosphorylated p95 fractionated with p185 in the particulate fraction, which contains the plasma membranes. p95 was further shown to be tyrosine-phosphorylated by first immunoprecipitating with antiphosphotyrosine antibodies and then probing the Western blot with anti-neu(C) (data not shown).

p95 Corresponds to Levels of Soluble ECD Released from Different Cells. To examine the relationship of p95 to soluble ECD, their levels were compared in different cells under varied conditions. The basal levels of ECD and cellular p95HER-2/neu were first examined in two cell lines that overexpress HER-2/neu, BT474 and the ovarian carcinoma cell line SKOV-3, which was previously reported to produce low levels of ECD (13). The amount of p95 relative to p185 and to cell protein was greatly elevated in BT474 cells, and correspondingly, the ECD in the extracellular medium from BT474 cells, detected with anti-neu(N), was enhanced by >10-fold compared to the SKOV3 cells (Fig. 4).

Shedding of several membrane proteins is rapidly and transiently induced by phorbol ester tumor promoters (44, 45). Although short-term treatment with tumor promoters does not induce HER-2 shedding (31), chronic administration of the phorbol ester TPA synergizes with chloroquine to stimulate release of soluble HER-2.[6] To determine whether p95 and ECD were coordinately regulated, TPA (500 nM) and chloroquine (50 μM) or the control vehicle were added to the culture media of BT474 and SKOV3 cells. At 24 h, the ECD levels in the extracellular media and p95 levels in the cell extract were analyzed. Soluble ECD was elevated severalfold in the conditioned

[6] T. A. Christianson, Y. J. Lin, and G. M. Clinton, unpublished observations.

3

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A Probe: α - neu (N)

p110>

0 1 10 20 40

TAP I (µM)

B Probe: α-neu (C)

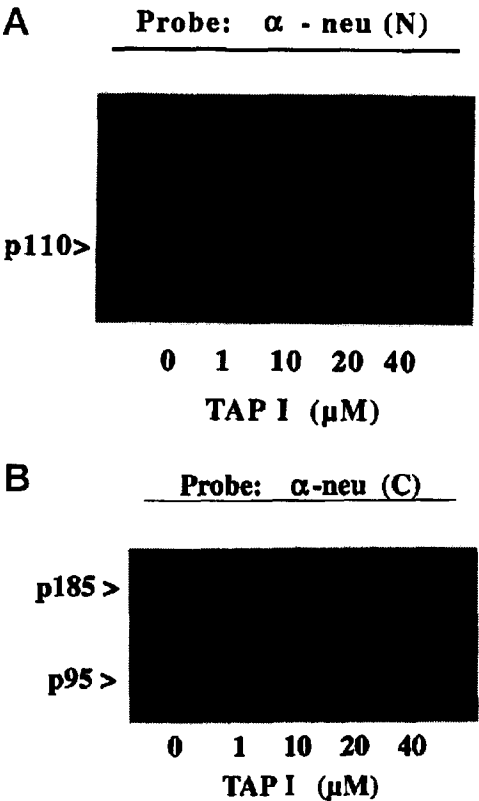p185 >

p95 >

0 1 10 20 40

TAP I (µM)

Fig. 5. p95 and ECD are inhibited by the hydroxamic acid TAPI. BT474 cells in serum-free medium were treated for 24 h with the control vehicle or with 1, 10, 20, and 40 µM TAPI (a gift from Immunex). *A*, the concentrated, conditioned media, normalized to the amount of cell extract, were analyzed by Western blotting with anti-neu(N). Similar results were obtained when 5 µg of protein from the conditioned media from each culture were analyzed. *B*, 20 µg of cell proteins were analyzed by Western blotting using anti-neu(C).

medium from stimulated BT474 cells and SKOV3 cells, whereas p95 was up-regulated ~3-fold in BT474 cells (Fig. 4). Overexposure of the immunoblot revealed that p95 in SKOV3 cell extracts was also stimulated about 3-fold by TPA and chloroquine (data not shown).

A Metalloprotease Inhibitor Depresses Levels of p95 and ECD from BT474 Cells. The shedding of diverse transmembrane proteins has been found to be inhibited by hydroxamic acid-based compounds, which are potent metalloproteinase inhibitors (46–48). We, therefore, tested the effects of different concentrations of the hydroxamic acid TAPI (47) on shedding of HER-2/neu ECD and on cell levels of p95. TAPI at 0–40 µM was added to cultured BT474 cells for 24 h, the ECD in concentrated conditioned media was analyzed by immunoblotting with anti-neu(N), and p95 and p185 were examined in cell extracts using anti-neu(C). The results in Fig. 5 show that production of ECD was partially inhibited at 1 µM TAPI and maximally inhibited by 10 µM TAPI. A residual amount of ~10% of the ECD resisted inhibition by 40 µM TAPI. The level of truncated p95 in the cytoplasm was also inhibited by TAPI, with little or no effect at 1 µM and maximal inhibition at 10 µM (Fig. 5). In three separate experiments, 1 µM TAPI inhibited ECD and p95 levels by ≤50%, and in all cases, maximum inhibition was achieved by 10 µM TAPI. No change in p185HER-2/neu levels could be detected in cells treated with TAPI or when shedding was stimulated by TPA and chloroquine (Fig. 4), probably because proteolytic processing of p185 is constitutive and limited, with ~20% converted into soluble HER-2/neu in 2 h (13).

Detection of p185 and p95 HER-2/neu in Breast Cancer Tissue. Tumor tissues were homogenized, fractionated, and examined for HER-2/neu proteins by Western analysis. The membrane-enriched but not the soluble fraction (data not shown) from some tumor tissues contained the full-length product, p185, and the truncated p95HER-2/neu protein that comigrated with HER-2/neu proteins from the control 17-3-1 cells (Fig. 6). In addition, p95, along with p185, was detected in two of eight ovarian cancer tissues (data not shown). Initial analyses of several breast cancer tissues revealed distinct expression patterns of p95 and p185. One group had no detectable p185 or p95 (Fig. 6, samples *39* and *69*). A second category of specimens expressed both p185 and p95 (Fig. 6, samples *60, 53, 04,* and *22*). An additional group contained p185 with relatively little or no p95 (Fig. 6, samples *40, 58, 38, 57, 17,* and *75*). As observed in previous studies, some samples were p185 rich (Fig. 6, samples *04, 22, 57, 17, 75*). The samples that were characterized as highly positive for p185 were initially identified by immunoassay values of >400 units (see "Materials and Methods" and Fig. 6 legend). The results of the Western analysis suggested that the tumors were heterogeneous with respect to HER-2/neu protein products and that they could be subdivided based on the presence or absence of p95.

Western analysis of 161 breast cancer samples revealed that 22.4% were p95 positive. The p185-positive samples were further subdivided into highly positive or HER-2-rich specimens, based on comparisons with HER-2/neu-overexpressing samples, identified by immunoassay and comparisons with the control 17-3-1 extract, as described in "Materials and Methods." The highly positive p185 samples represented 21.7% of the total samples. All samples that expressed p95 were also positive for p185, although 65% of p185-positive samples did not contain p95. Of the p95-positive samples, 63.9% were also highly positive for p185, and 36% had low p185 levels.

Relationship between p95-positive, Highly p185-positive, and Other Prognostic Factors. The proportion of 78 node-positive breast cancer patients expressing p95 was higher than the proportion of node-negative patients expressing p95 ($P = 0.032$); p185-rich samples had no significant association with node status (Table 1).

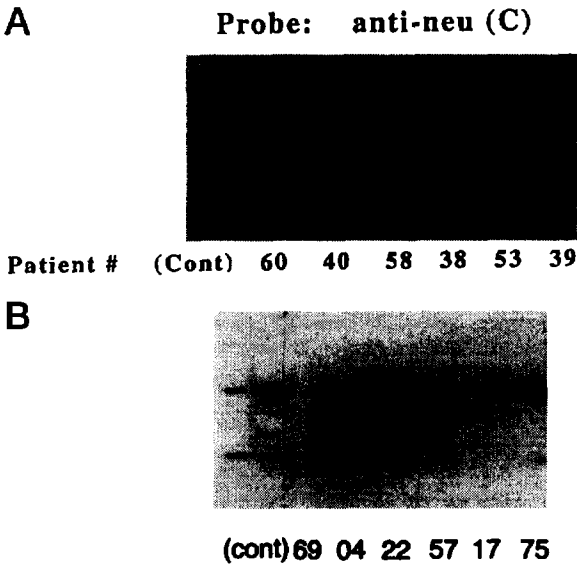Neither p95-positive nor p185-rich samples correlated significantly

A Probe: anti-neu (C)

Patient # (Cont) 60 40 58 38 53 39

B

(cont) 69 04 22 57 17 75

Fig. 6. Western blotting analysis of 12 breast cancer tissues. Human intraductal breast cancer tissues were fractionated, and 20 µg of protein from 12 patients were subjected to Western blotting with anti-neu(C) as described in "Materials and Methods." The control lane contained 3 µg of protein from transfected 3T3 cells, 17-3-1. The positions of p185 (*top band*) and p95 (*bottom band*) are marked in the control 17-3-1 sample in *B*. A photograph of the film that was exposed to the membrane for 20 min (*A*) and for 5 min (*B*) is shown. HER-2/neu immunoassay values were: <100 units for samples *60, 39,* and *69*; 389 units for sample *40*; 258 units for sample *58*; 302 units for sample *38*; 200 units for sample *53*; 2,000 units for sample *04*; 10,000 units for sample *22*; 1000 units for sample *57*; 550 units for sample *17*; 674 units for sample *75*.

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Table 1 *Relationship between p95-positive and highly p185-positive status and other prognostic factors[a]*

Factor	% p95 positive	P	% p185 highly positive	P
Nodes		.032		NS[b]
Positive (78)	29.5		24.4	
Negative (63)	14.3		22.2	
Tumor size (cm)		NS		NS
≥3 (54)	27.8		22.2	
<3 (79)	17.7		21.5	
ER		NS		NS
Negative (37)	32.0		29.7	
Positive (117)	19.7		17.9	
PR		NS		NS
Negative (59)	23.7		20.3	
Positive (95)	22.1		23.2	

[a] 161 samples were examined by Western analysis. See "Materials and Methods" for a description of patient material used and methods of analysis. Not all samples had information for the factors examined.
[b] NS, not significant.

with other factors known to predict poor prognosis (49), including ER and PR negativity and tumor size of ≥3 cm (Table 1).

Influence of p95 in the Highly p185-positive Group. We questioned why a similar percentage of node-positive and node-negative patients were p185 rich (24.4% *versus* 22.2%, Table 1), whereas p95 was associated with node-positive patients (65.7% of the p185-rich samples contained p95). We, therefore, examined whether the presence or absence of p95 in the specimens that overexpressed p185HER-2/neu affected the relationship with lymph node status (Table 2). The highly p185-positive samples that contained p95 (*n* = 21) had a significantly higher association with metastasis to the lymph nodes, whereas the highly p185-positive samples that were negative for p95 (*n* = 11) were associated with lymph node-negative patients (*P* = 0.017).

DISCUSSION

We identified an NH_2-terminally truncated HER-2/neu product of M_r ~95,000, which was detected by Western blotting and by immunoprecipitation with antipeptide antibodies against the COOH terminus but did not react with monoclonal antibodies against the NH_2 terminus of p185HER-2/neu. p95 has kinase activity evidenced by its self-phosphorylation when p185 was cleared from the cell extract prior to immunoprecipitation with anti-neu(C) (Fig. 1). Several controls and extraction procedures were conducted to rule out that p95 was created by an *in vitro* degradation artifact. Cells extracted with protease inhibitors had only two major cytoplasmic HER-2/neu proteins, p95 and p185, with no indication of smaller degradation products. p95 levels were not affected by procedures that would eliminate the activity of proteases, including direct extraction of cells in boiling 10% SDS-containing buffers.[7]

One mechanism described previously for generation of NH_2-terminally truncated RTKs is proteolytic release of their ECDs (28–31). Several lines of evidence point to the production of p95HER-2 in cultured cells by endoproteolytic processing. Its presence in 17-3-1 cells transfected with HER-2/neu cDNA argues that p95 is a proteolytic product rather than the product of an alternative transcript. Furthermore, the levels of p95 and soluble HER-2 ECD released from cultured cells were correlated. First, both p95 and ECD levels were low in SKOV3 cells compared to BT474 cells (Fig. 4). Second, augmentation of both p95 and ECD by long-term (24-h) treatment with TPA and chloroquine (Fig. 4) further indicated that the truncated HER-2 products were generated through a common pathway. Al-

[7] T. A. Christianson and G. M. Clinton, unpublished data.

though the mechanism for this stimulation was not examined, long-term exposure of cells to TPA has been found to enhance internalization of RTKs (50), whereas chloroquine, an agent that alters the pH in endosomes and lysosomes, may inhibit complete proteolytic breakdown or alter RTK trafficking (51). Finally, p95 and ECD were both inhibited by addition of the hydroxamate compound, TAPI, to intact cells, and both were maximally inhibited by ≤10 μM TAPI (Fig. 5). The strong inhibition by TAPI indicates that most of the ECD and p95 in BT474 cells were generated by a metalloprotease (46, 47) and that this class of inhibitors may be effective in controlling shedding in breast cancer patients. Although p95 and shedding were modulated under several different conditions, changes in the p185 levels could not be detected. Unlike several transmembrane proteins that only shed when induced by TPA, proteolytic shedding of p185 occurs continually at a low basal level (11, 12), with only ~20% converted into soluble ECD in 2 h (13). The truncated cell protein of M_r ~95,000 described here was somewhat larger than the expected M_r 75,000–80,000 for the cytoplasmic remnant of the M_r ~105,000–110,000 ECD. p95 or the ECD might migrate anomalously in gels because the site of cleavage for ECD shedding is not known. Although our studies showed that the ECD and p95 are coordinately produced in culture by proteolytic activity that is sensitive to a metalloprotease inhibitor, it is not yet known whether p95 levels in breast tumors will be directly coupled to serum ECD. In some cases, ECD may be the product of an alternative transcript (14), or the metabolism of p95 may vary in different cells. Future studies aimed at testing cancer tissue and serum from the same patients will be required to evaluate whether serum ECD correlates with tissue p95 *in vivo*.

A HER-2/neu product of the same size, M_r 95,000, in transfected 3T3 cells, cultured breast carcinoma cells, breast cancer tissue, and ovarian cancer tissue suggests that a similar proteolytic processing event may occur in the different cells. However, p95 was not detected in all cells and tumor tissue that contain p185. Two nontumorigenic breast epithelial cell lines had no detectable p95 (Fig. 2). In addition, the SKOV3 ovarian carcinoma cells, which overexpress p185, had a disproportionately low amount of p95 (Fig. 4). These observations indicate that production of p95 is regulated. The cells with variable levels of truncated HER-2/neu products may differ in the amount of the relevant protease activity or the protein substrate may have an altered conformation affecting sensitivity to proteolytic cleavage.

p95HER-2/neu has properties that suggested a rationale for examining its association with prognostic factors in breast cancers. It has kinase activity, is tyrosine-phosphorylated, suggesting its activity in signaling, and is truncated from its NH_2 terminus. Oncogenic signaling by HER-2/neu is known to depend on its level of kinase activity (33–35). Because p95 was at 100% of p185 in some breast cancer samples, it may impact the amplitude of the kinase signal. Moreover, an NH_2-terminally truncated kinase domain such as p95 is expected to emit a constitutive signal by analogy to results with engineered deletions of the ECD from the HER-2/neu product (32–36). Taken

Table 2 *Relationship between highly p185-positive samples that are p95 negative versus p95 positive and node status*

	Highly p185 positive[a]	
	p95 positive (*n* = 21)	p95 negative (*n* = 11)
Node positive	71.4%[b]	27.3%
Node negative	28.6%	72.7%

[a] The highly p185-positive group (*n* = 32) was divided into those that contained p95 (*n* = 21) and those that were p95 negative (*n* = 11).
[b] The samples that contained p95 had a significantly higher association with node-positive patients (15 of 21), and those that were p95 negative correlated with node negative patients (8 of 11; *P* = 0.017).

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together, these results suggest that p95 will elevate the kinase signal in some patients and could, thereby, be associated with more aggressive tumor growth. Although this was the biological rationale used in our study, p95 could also mark tumors that express a particular protease that is relevant to breast cancer pathology.

In this study, 161 breast cancer tissues were homogenized, fractionated, and analyzed by Western blotting, a technique required to distinguish p185 from the truncated cytoplasmic protein. A comprehensive study conducted by Tandon et al. (52) also used Western analysis of breast tissue extracts, but they evaluated only the full-length product, p185. In agreement with their results, we also found p185 expressed frequently in breast tumors, with a subpopulation of 21.7%, compared to their proportion of highly positive scores (16%). Western blot technique has been reported to be the most prone to false-negative errors because of dilutional artifacts introduced by extracellular matrix from stroma-rich cancers (29). For our study, homogenized tissue was fractionated into a membrane-rich fraction, from which relatively insoluble extracellular matrix proteins would tend to be eliminated.

One of the major findings of this study is that breast cancers, which express HER-2/neu, are heterogeneous with respect to protein products. Importantly, the distinct products, p95 and p185, were differentially associated with node status. Although the group that overexpressed p185 did not associate with node status (Table 1), those that were p185-rich and contained p95 were significantly associated with lymph node metastasis (Table 2). This may help explain why several previous studies, which have attempted to show association with lymph node metastasis based on assays of p185 protein overexpression or HER-2/neu gene amplification, have yielded inconsistent results (see Ref. 6). A biological explanation for our findings is that loss of the ECD regulatory region from the p95 kinase combined with amplified p185 signal in primary breast tumor cells could promote their metastasis to the lymph nodes.

p95-positive or highly p185-positive samples did not correlate with other prognostic markers in this study, including tumor size or hormone receptor status. Although no consistent correlation with tumor size has been detected, other studies have reported association of HER-2/neu overexpression with ER and PR negativity (6, 52, 53). The smaller numbers of samples examined in our studies may explain the failure to reach statistical significance. Moreover, in contrast to our study, the relationship between HER-2 overexpression and hormone receptor status was examined in a subgroup of high-risk patients or in groups that were stratified by levels of hormone receptors (52, 53).

In conclusion, it has been difficult to understand why HER-2/neu overexpression occurs in tumor tissue from both node-negative and -positive patients, yet it is a strong prognostic marker only in node-positive patients (5–9, 52). Our findings, which need to be validated in a different patient population, indicate that p95 is preferentially found in HER-2/neu-positive patients with lymph node involvement. It is possible that higher expression of p95 is a critical factor that helps explain the increased prognostic significance of HER-2/neu in node-positive patients. It will be of interest and importance to examine the prognostic significance of p95, particularly in node-negative patients.

ACKNOWLEDGMENTS

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